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Changes in S100 proteins identified in healthy skin following low level electrical stimulation: a possible relevance for wound healing

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50-WORD ABSTRACT

Targeted electrical energy applied to wounds has been shown to improve wound-healing rates. However, the mechanisms are poorly understood. We show that electrical energy stimulation applied continuously for 48 hours to the skin of healthy patients has the effect of modifying gene expression in a number of identified genes.

ABSTRACT

Objective Targeted electrical energy applied to wounds have been shown to improve wound-healing rates. However, the mechanisms are poorly understood. The aim of this study was to identify genes that are responsive to electrical stimulation (ES) in healthy subjects with undamaged skin.

Approach To achieve this objective we used a small non-invasive, ES medical device to deliver a continuous specific set sequence of electrical energy impulses over a 48-hour period to the skin of healthy volunteers and compared gene expression by microarray analysis.

Results Application of this specific electrical impulse resulted in differential expression of 105 genes, the majority of which were downregulated. Our post-microarray analyses revealed there was commonality with a small number of genes that have previously been shown to be upregulated in skin wounds including venous leg ulcers.

Innovation

The identification of the differential expression in this subset of genes in healthy subjects provides new potential lines of scientific enquiry for identifying similar responses in subjects with slow or poorly healing wounds.

Conclusion

The specific sequence of electrical energy stimulation applied continuously for 48 hours to the skin of healthy patients has the effect of modifying gene expression in a number of identified genes.

INTRODUCTION

The use of externally applied electrical energy to promote the healing of complex wounds is a concept that was introduced more than 40 years ago [1]. There are various types of electrical stimulation (ES) devices but despite variation in application mode, dose and duration of therapy, the majority of trials show significant improvement in wound healing or wound area reduction compared to control treatment or standard care (reviewed by [2, 3]). The use of continuous direct current of 200-800 μA and pulsed current improved healing of chronic wounds in a number of studies [4-9]. However, the mechanisms of ES-mediated wound healing *in vivo* are poorly understood with a number of different explanations provided for the clinical outcomes reported.

A recent systematic review concluded that the ideal ES device needs to be non-invasive, portable, cost-effective and provides minimal interference with patients' daily life [3]. In addition, a device that delivers a programme of fixed parameters and duration is important in order to standardise findings and to make comparisons and draw conclusions from studies or clinical applications that use it. The device used for this study is a class IIA medical device (under medical directive 93/42/EEC, which has full Medical Devices Directorate approval under ISO 13485:2003). The device delivers a proprietary frequency of pulsed electrical energy at the microcurrent level delivered directly to the skin via two off-the-shelf treatment electrode pads placed directly onto intact skin for a duration of 48 hours. Knowledge of the existence of ES-responsive genes might provide new potential lines of scientific enquiry for identifying similar gene responses in subjects with slow or poorly healing wounds.

CLINICAL PROBLEM ADDRESSED

The identification of how ES can regulate or modify gene expression in skin is critical in improving the understanding of why ES treatment improves healing rates in chronic wounds in some individuals and not in others. If we could identify the ES-responsive genes it might be possible to measure the expression in responders and non-responders to predict treatment efficacy in different patient groups and to select ES treatment parameters that yield the best wound healing outcomes.

MATERIAL AND METHODS

Study design and participants

The study was an investigator-initiated, double-blinded, randomized, placebo-controlled trial undertaken at Bispebjerg Hospital, Copenhagen, Denmark. The study was reported to the Danish register (*Datatilsynet*), and was performed in accordance with Danish law (*Lov om behandling af personoplysninger*). This included an approval from the Regional Ethical Committee for the Hospital Region of Greater Copenhagen (Region H) (H-3-2012-158). Only healthy male Caucasians, aged 20-30 years old, a BMI of between 19 and 25, non-smokers, no history of cancer, diabetes or vascular disease, and who undertook similar levels of moderate physical activity per week were recruited. Two ES devices identical in appearance (one active and one placebo) were attached to pairs of electrodes identical in appearance, were placed on intact healthy skin of opposite buttocks of 12 healthy male subjects. The electrode pads of each device were 10 cm apart and there was a 20 cm gap between the electrode pads of the different devices (Fig. S1B). Only a third independent person had knowledge of which device was active, thus the study was double-blinded. The placebo devices were identical in appearance and function to the activated devices. After 48 hours full-thickness skin biopsies of 4-mm diameter were excised from a region midway between the two electrodes.

Comparative microarray

RNA was isolated from skin biopsies and cells using TRIzol reagent (Invitrogen) following manufacturers' protocols as previously described[10]. RNA concentrations and integrity were measured using Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA was amplified and hybridized to Human Genome U133 2.0 GeneChip arrays (Affymetrix). Microarray data are available in the ArrayExpress database under accession number E-MTAB-3935. Technical quality control and outlier analysis was performed with dChip (V2005)[11] using the default settings. Background correction, quantile normalization, and gene

expression analysis were performed using RMA in Bioconductor[12]. To assess if ES altered gene expression in the skin, an ANOVA linear model was employed to evaluate where the variation within the data set came from (Partek Genomics Solution version 6.5, Copyright 2010, Partek Inc., St. Charles, MO, USA). Differential expression analysis was performed for subjects that were exposed to ES using a paired model in Limma using the functions lmFit and eBayes[13]. A gene list of differentially expressed genes was formed from probe sets that had >1.5-fold change, $p<0.05$.

Data analysis

Gene ontology analysis using DAVID (Database for Annotation, Visualization and Integrated Discovery) and Ingenuity Pathway Analysis (Qiagen) showed there is an enrichment of RAGE-binding proteins: S100A7, S100A8, and S100A9. A comparison was performed with wound-regulated genes in a microarray data set generated by Cooper *et al.* [14] under the ArrayExpress accession number E-MTAB-3836[14] and with genes differentially regulated in venous leg ulcer (VLU) wound edge as described[15].

RESULTS

The study comprised of 12 participants, of which nine received one activated and one placebo ES device and three participants received only placebo devices (Fig. 1). The device delivered a series of pre-set programs in which there is varying amplitude (40-500 μA), frequency (10-900 Hz) and polarity (alternating every 0.1 seconds). The electrical pulses were delivered as repeated 240-minute treatment sessions with a two-hour resting period between each in the first 24 hours and a four-hour resting period between each in the second 24 hours (Fig. S1). RNA was isolated from full-thickness skin biopsies and analysed by microarray. Unbiased statistical analyses showed that one of the 24 samples was an outlier and was excluded from subsequent analyses (Fig. S2A). Both principal component analysis and linear model evaluation suggested that the largest source of variation in global gene expression changes was from differences between individuals (Fig. S2B). No significant differential expression was detected between biopsies from treatment with two placebo devices in any of the 3 control participants. A within-subject analysis of ES treated versus placebo treated skin produced a significant variation score of 1.47, as illustrated by the 15 most differentially regulated (Fig. S2C). Thus, alterations in levels of gene expression levels could be attributed to the 48-hour ES treatment.

To accommodate the large variation due to individuals a paired test was used, resulting in 105 annotated genes which have significant differential regulation of 1.5-fold ($p<0.05$) in ES treated skin compared with placebo treatment (listed in Supplementary Table 1). A volcano plot of the microarray data shows that the majority of these differentially regulated genes were reduced in expression, with the exception of two genes (Fig. 2A).

We subjected this list of ES regulated genes to unbiased functional annotation analyses using DAVID (Database for Annotation, Visualization and Integrated Discovery) and by IPA (Ingenuity Pathway Analysis). Functional gene ontology analysis showed that there was an overrepresentation of genes in particular cellular compartment, including focal adhesion (*ANXA6*, *PDIA3*, *TPM4*, *LPP*), protein-DNA complex (*CTNNB1*, *HNRNPK*, *JUP*),

and endoplasmic reticulum-Golgi intermediate compartment membrane (*TMED9*, *RAB2A*, *ERGIC1*), and in genes encoding ribonucleoprotein (*TEP1*, *HNRNPH1*, *MRPS16*, *MRPL30*), gene activators (*SUPT4H1*, *SMARCA2*, *MAX*, *RORC*, *EBF1*), and RAGE (receptor for advanced glycation end products)-binding proteins (*S100A7*, *S100A8*, *S100A9*) (see Supplementary Table 1). Pathway analysis showed that these ES-regulated genes were implicated in canonical pathways, including regulation of macrophages production of IL-12, nitric oxide and reactive oxygen species, and IL-17A in psoriasis (Fig. 2B). Next, we compared the list of ES regulated genes to two publically available sets of array data that identified genes regulated by wounding. First, we compared our data set to over 2600 probe sets differentially regulated 24 hours after wounding *in vivo* identified in a mouse skin injury model[14]. In common were 25 genes that were upregulated in wounding but downregulated with ES and one gene (*RAD23B*) was similarly downregulated in both microarray studies (see Table 1). Next, we compared the list of ES regulated genes to the top 50 most upregulated and top 50 most downregulated genes identified in the wound edge of venous leg ulcers (VLUs)[15]. All three *S100* genes and *SERPINB4* were upregulated in the VLU wound edge but were downregulated in ES-treated skin (see Table 1).

DISCUSSION

The primary finding of the current study is that ES delivered with a pre-set fixed programme and duration to healthy human skin reduces the expression of a specific set of genes that have been found to be upregulated in skin inflammation. This study used healthy volunteers instead of patients with chronic wounds primarily because of the risk of the biopsy procedure could have adverse effects on the healing of the wound. We do not know from the results of this study, but can hypothesise, that the same ES device applied in the same manner would have the same effects on healthy wound edge skin adjacent to a chronic wound. However, many patients with chronic, poorly and non-healing wounds commonly present with

co-morbidities so this hypothesis would have to be fully tested and the results compared with the current study in order to draw a safe conclusion.

The duration of the treatment of 48 hours was chosen to investigate any immediate differential gene expression because it is one application of the device's full delivery cycle. As the device was applied over the buttocks, we suggest that 48 hours was an acceptable length of time for participants to withhold from showering, bathing and activities that could cause excessive sweating. Analysis of the sources of variation reinforced the fact that there is extensive variation between participants, but did indicate that ES significantly affected the expression of specific genes. Whilst the variations of age, gender, smoking, activity levels and no medication were controlled for, other variations in the participants' life style (e.g. activity and diet) or underlying medical conditions were unknown. However, the participants were all healthy young individuals without any signs of abnormal conditions or diseases. The participant profiles of the most regulated genes varied between participants, suggesting that some of the participants could be viewed as either responders or non-responders to the ES treatment.

Functional annotation analysis of the 105 ES-regulated genes showed that there is an enrichment of RAGE-binding proteins: S100A7, S100A8, and S100A9. We were able to compare our findings with only two other publically available array data sets produced from wound-related studies [14, 15], however the comparisons confirmed that S100 genes were upregulated in both acute and chronic wounds. These proteins are expressed at low levels in keratinocytes in healthy skin but are upregulated upon wounding. The expression of S100 genes has been shown to be crucial for re-epithelialization of the wound[16], recruitment of inflammatory cells[17, 18], and regeneration of the hair follicle[19], and regulating keratinocyte growth and differentiation[20], which can promote wound healing. Controversially, S100 proteins are also readily detected in wound exudates[21, 22], upregulated in chronic wounds[15, 23] and psoriasis[24, 25], which suggest their expression is a biomarker for non-healing wounds. Overexpression of S100 genes in HaCat keratinocytes impaired collective migration in the cell

sheet immediately behind the migrating front in an *in vitro* scratch wound assay, and staining of β-catenin showed cells adjacent to gaps in the cell sheet had no positive staining in the periphery but accumulated cell junction proteins in the cytosol when S100 genes were over expressed (data not shown).

It is thought that a prolonged inflammatory phase and the increased expression of proteolytic enzymes prevent wounds from progressing into the proliferative phase, and are major factors that contribute to wounds becoming non-healing[26]. Pathway analysis indicated that the ES application dampened the release of pro-inflammatory cytokine interleukin-12, nitric oxide and reactive oxygen species by macrophages known to be responsible for the breakdown of connective tissues (reviewed by[27]). Reduction of S100A7, S100A8, and S100A8 and activation of macrophages by ES could potentially improve wound healing by dampening these pathways in chronic wounds.

In summary, for future studies it could be hypothesized that ES mediated healing of chronic wounds could be achieved via down-regulation of certain gene pathways that are known to compromise wound healing and which are upregulated in skin wounds, thus enabling an improved rate of wound repair.

Limitations of the study

This study was performed on a small number of participants. We cannot make an inference from this study that if the same ES device was applied in the same manner to the periwound skin of a poorly healing wound it would have the same effect as on skin of healthy volunteers. Patients with chronic, poorly and non-healing wounds commonly present with co-morbidities so this hypothesis would have to be fully tested and the results compared with the current study with larger subject numbers with different wound types in order to draw a safe conclusion. There are no known or anticipated or known side effects from this electroceutical application. The product used is CE marked for safety and is accredited as a Class IIA medical device under the directorate ISO13485.

A single type of ES device was used for this investigation delivering a fixed pulse sequence and duration of ES. Therefore, we do not know if the same changes in gene expression reported here would be seen if a different set of ES parameters were used.

Further areas of study

The identification of the changes in gene expression, albeit in healthy skin provide potential novel therapeutic targets for the development of a treatment for individuals who present with delayed skin healing. However, this study used the skin on the buttocks of healthy individuals so findings may be different in different areas of the body where skin thickness varies. Therefore, this too would have to be ruled out as a significant variant.

Identifying higher levels of these proteins may help to identify the patients in this group who are likely to have healing problems. However, as a single type of ES device was used for this investigation delivering a fixed frequency of pulsed electrical energy and duration of ES further investigation is required to see if the same changes in gene expression are caused using a different set of ES parameters. The UK's National Health Service (NHS) managed an estimated 2.2 million patients with a wound during 2012/2013, equivalent to 4.5% of the adult population [28]. The annual cost to the NHS attributable to wound management and associated comorbidities was estimated at £5.3 billion[28]. Our study is a first step towards a mechanistic understanding of the potential benefits of electrical stimulation for improving skin healing in pathological conditions.

INNOVATION

This study provided an insight and a line of further investigation into a possible mechanism by which ES improves the rate of healing in poorly healing wounds, whereby genes upregulated during inflammation in wounds are inactivated by the ES application. We identified 105 genes in skin that are regulated, most of which were downregulated, by application of ES for 48 hours. The identification of these genes, albeit in healthy skin, provides potential novel therapeutic targets for the development of a treatment for individuals who present with delayed skin healing. Identifying higher levels of these proteins may help to identify the patients in this group who are likely to have healing problems.

KEY FINDINGS

- A defined and set programme of electrical stimulation (ES), delivered to the skin regulates 105 genes in the skin, the majority of which are downregulated by the treatment.
- Our post-microarray analyses revealed there was commonality with genes that have previously been shown to be upregulated in complex wounds in skin.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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ABBREVIATIONS AND ACRONYMS

ES electrical stimulation

VLU venous leg ulcer

TABLES

Table 1. List of genes common with those differentially regulated in skin 24 hours post wounding and in VLU wound edge.

Gene Symbol	ES device vs. placebo **(fold change)**	Acute wound vs. control **(fold change)**	VLU wound edge vs. control **(fold change)**[15]
SERPINB4	-3.62	-	94.96
S100A7	-3.37	-	105.80
EIF5A	-2.52	1.62	-
S100A8	-2.26	90.42	95.11
A02B1	-2.24	1.47	-
PDAP1	-2.17	1.52	-
S100A9	-2.16	97.95	119.00
LYZ	-2.14	1.24	-
SET	-2.09	1.48	-
VCAN	-1.98	8.59	-
CPR124	-1.93	2.72	-
CD47	-1.92	1.60	-
RAD23B	-1.92	-1.26	-
SPP1	-1.90	7.52	-
CALD1	-1.88	1.52	-
ARPC4	-1.87	1.77	-
LCN2	-1.81	44.12	-
PDIA3	-1.67	1.56	-
ERGIC1	-1.67	1.70	-
RNF125	-1.66	1.27	-
KRT17	-1.64	2.72	-

KRT6B	-1.63	146.32	37.66
APP	-1.62	-	-9.17
TPM4	-1.55	1.70	-
RBM3	-1.53	1.53	-
SOX7	-1.53	1.23	-
GNB4	-1.52	1.51	-
AQP3	-1.52	2.80	-
GNG12	-1.50	1.36	-

FIGURE LEGENDS

Figure 1. Flow chart of study to identify genes in skin that are regulated by ES.

Figure 2. ES treatment to healthy skin leads to downregulation of gene expression.
(A) Volcano box plot to illustrate the expression changes of all genes. The 105 genes whose expression was significantly altered ≥1.5-folds, $p<0.05$ are shown in the top left and top right boxes. (B) Top 10 canonical pathways identified in ES-regulated genes in skin by Ingenuity Pathway Analysis.

Table 1. Genes common with those differentially regulated in skin 24 hours post wounding **and in VLU wound edge**.

Supplementary Figure 1. ES application to skin of healthy participants.
(A) The ES device. (B) Two ES devices were applied to the skin overlying the buttocks (one on each side). The electrode pads of each device were 10 cm apart and there was a 20 cm gap between the electrode pads of the different devices. (C) Program specification of the ES device.

Supplementary Figure S2. Variation between individuals.
(A) D-chip analysis of samples obtained from the 12 participants of the study. *Warning for sample from participant 8's ES treatment as an array outlier, which was subsequently excluded from further analyses. (B) Principal component analysis (PCA) was used to provide a statistical summary of the array samples, and the first three principal components are shown. C = control; MC = microcurrent (ES). (C) The top 15 differentially regulated genes (as ranked by fold change) of the 8 participants who were treated with a placebo and the ES device. Note that SERPINB4 expression in participant 1 is not included in the graph but it showed a fold change of over -90 with ES treatment.

Supplementary Table 1. List of 105 genes in skin regulated differentially by 48 hours of treatment with the ES device.

Supplementary Table 2. Annotation clusters of genes differentially regulated by an externally applied electrical stimulation on healthy skin.

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